Hepatocytes corrected by gene therapy are selected *in vivo* in a murine model of hereditary tyrosinaemia type I

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Current strategies for hepatic gene therapy are either quantitatively inefficient or suffer from lack of permanent gene expression. We have utilized an animal model of hereditary tyrosinaemia type I (HT1), a recessive liver disease caused by deficiency of fumarylacetoacetate hydrolase (FAH), to determine whether *in vivo* selection of corrected hepatocytes could improve the efficiency of liver gene transfer. As few as 1,000 transplanted wild-type hepatocytes were able to repopulate mutant liver, demonstrating their strong competitive growth advantage. Mutant hepatocytes corrected *in situ* by retroviral gene transfer were also positively selected. In mutant animals treated by multiple retrovirus injections >90% of hepatocytes became FAH positive and liver function was restored to normal. Our results demonstrate that *in vivo* selection is a useful strategy for hepatic gene therapy and may lead to effective treatment of human HT1 by retroviral gene transfer.

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The liver plays a central role in the pathophysiology of many inborn errors of metabolism, because it is the major site of catabolic, synthetic and detoxification reactions[1,2]. Many of the known enzyme deficiency disorders are treatable by orthotopic liver transplantation and therefore would potentially also be amenable to liver gene therapy[3]. Unfortunately, current liver gene transfer protocols are not able to provide long-term transduction of the majority of hepatic tissue in human or animal trials. Retroviral gene transfer provides stable integration and long-lasting expression of therapeutic genes, but achieves correction of only a small percentage of hepatocytes *in vivo*[4]. In contrast, adenoviral vectors can infect 100% of liver cells *in vivo*, but the expression does not persist because of the episomal nature of the virus and immunologic rejection of infected cells[5,6]. We now describe *in vivo* selection as a strategy for hepatic gene therapy in a murine model of the human hereditary liver disease tyrosinaemia type I (HT1). This approach offers selection and enrichment of long term corrected hepatocytes *in vivo*.

HT1 is caused by deficiency of the enzyme fumarylacetoacetate hydrolase (FAH), the last step in tyrosine catabolism[7]. Humans with this condition suffer from progressive liver failure during infancy, renal tubular damage, porphyria like neurologic crisis and early development of hepatocellular carcinoma[8]. The accumulating substrate of FAH, fumarylacetoacetate (FAA) and its precursor maleylacetoacetate (MAA) are thought to be hepatotoxic and mutagenic — and are therefore likely to be responsible for the phenotype. We have generated a strain of mice, designated $Fah^{\Delta exon5}$, that are deficient in Fah by targeted disruption of exon 5 of the *Fah* gene[9]. Homozygous mutant mice die in the neonatal period due to hepatic dysfunction and have a phenotype analogous to the c^{14Cos} lethal albino mouse. The deletion of lethal albino mice contains the *Fah* gene[10,11]. Fah deficient mice have a grossly altered pattern of hepatic mRNA expression — particularly in genes responsive to cAMP, they suffer from severe hypoglycaemia, and the endoplasmic reticulum of hepatocytes is disorganized[11–13]. The neonatal lethality and misexpression of liver mRNAs in $Fah^{\Delta exon5}$ mice can be rescued by the drug 2-(2-nitro-4-trifluoro-methylbenzyol)-1,3-cyclohexanedione (NTBC), which blocks tyrosine catabolism upstream of FAH and therefore prevents the buildup of hepatotoxic metabolites[14,15]. When NTBC-rescued Fah^{-} adults are removed from the drug, they develop a phenotype similar to humans with HT1, including rapidly progressive hepatic dysfunction and death within 2 months after drug withdrawal. Unfortunately, the development of hepatocellular carcinoma is not prevented even with continuous life-long use of NTBC[15]. Current therapeutic strategies in human HT1 include dietary restriction of tyrosine and phenylalanine, the use of NTBC and orthotopic liver transplantation[14,16,17].

Recently it has been observed that the livers of human HT1 patients frequently contain discrete nodules with FAH enzyme activity, due to somatic reversion events[18]. FAH enzyme positive nodules were detected in 15/18 HT1 livers at transplantation[19]. Molecular studies demonstrated the correction of one of the disease-causing alleles in all such nodules. The

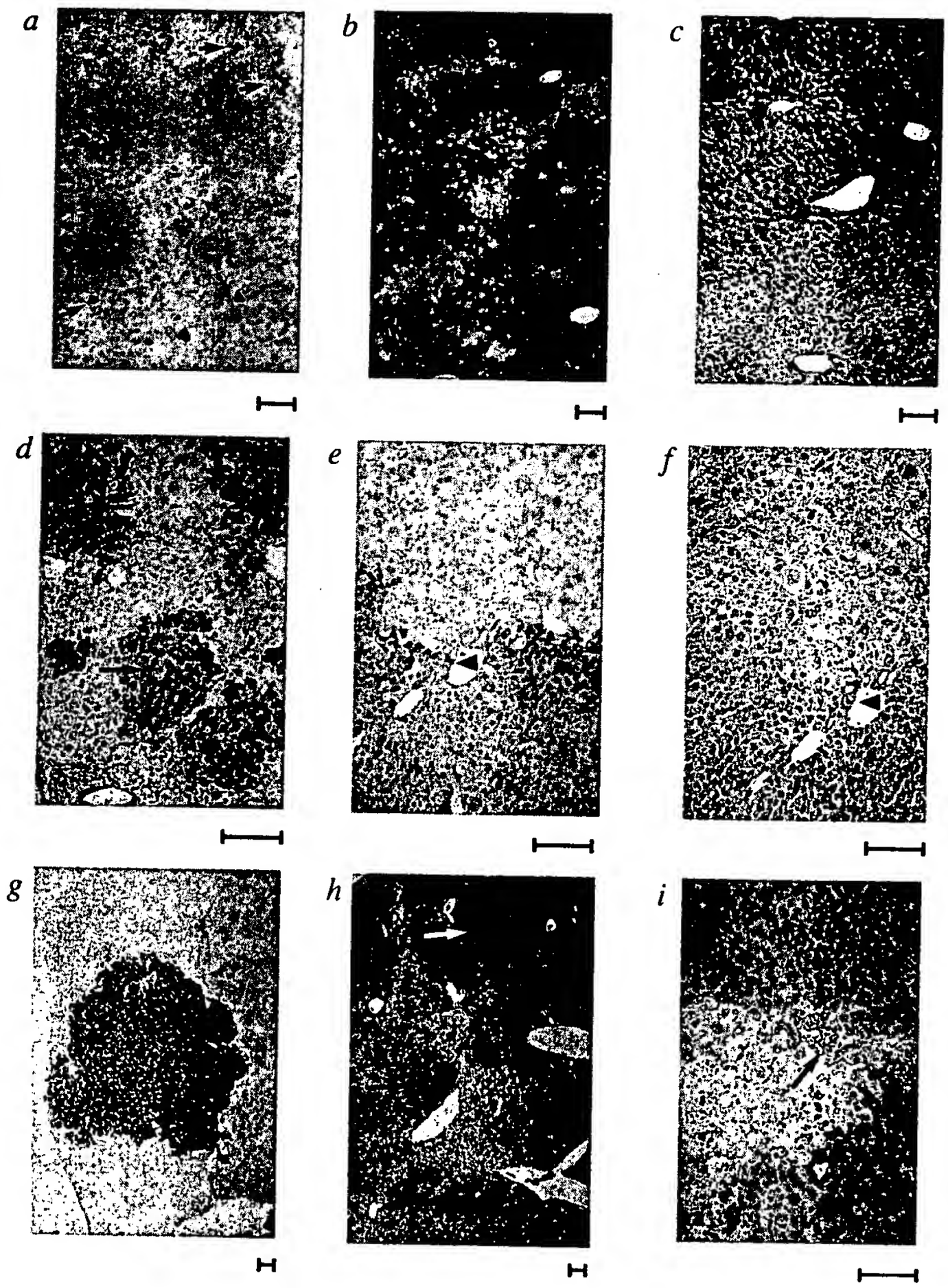

Fig. 1 Liver histology of transplanted and retrovirus treated Fah mutant mice. All panels except (*f*) represent immune histology with an Fah antibody. Dark areas are Fah positive. The size of the bar corresponds to 100 μm. *a*, Fah mutant transplanted with 10^6 wild-type cells, 2 months after transplantation. This animal was continuously treated with NTBC and only very few Fah positive cells (arrows) were found. *b*, Same as (*a*), but NTBC was discontinued after transplantation. About 90% of hepatocytes are Fah positive. *c*, Wild-type control mouse with positive staining in all hepatocytes. *d*, Mutant mouse off NTBC, 3 weeks after transplantation. Individual transplanted wild-type cells are forming clones (arrows). *e*, Adjacent Fah positive and negative areas in a transplanted mutant mouse. The Fah-deficient area (solid arrow) showed infiltration with inflammatory cells and marked variability in cell and nuclear size (dysplasia). Adjacent hepatocytes expressing retroviral Fah have normal morphology. *f*, Haematoxylin/eosin stain of the same area as shown in (*e*). For alignment, the small triangle indicates a portal area present in both (*e*) and (*f*). *g*, Repopulation nodule in mutant mouse transplanted with 100 donor cells. The nodule measures about 1.4 mm across. (*h*) Mutant animal injected with multiple doses of *FAH* retrovirus. The majority of hepatocytes express FAH, but there are clonal areas of high (white arrow), intermediate (black arrow) and low expression (open arrow). (*i*) Same as (*h*) but higher magnification. FAH deficient tissue shows dysplasia and inflammatory cells (arrow).

clonal appearance of FAH positive regions and the high prevalence of such reversions suggested the possibility of positive selection for FAH producing cells in HT1 livers. A precedent for such *in vivo* selection of hepatocytes has been observed in an albumin-urokinase transgenic mouse[20]. This transgene is toxic to hepatocytes and somatic loss of the urokinase gene in liver cells confers a selective growth advantage. This leads to complete repopulation of the liver with hepatocytes which have spontaneously deleted the transgene. In the urokinase transgenic system transplanted wild-type cells are strongly selected *in vivo*[21]. We reasoned that if Fah-expressing cells indeed had a growth advantage in $Fah^{\Delta exon5}$ mutant livers, this phenomenon could be exploited to achieve both long term stable expression and correction of the majority of liver tissue by *in vivo* retroviral gene transfer.

Here we show that transplanted Fah-expressing hepatocytes effectively repopulate the diseased liver of $Fah^{\Delta exon5}$ mutant mice. NTBC suppresses this repopulation, indicating that it is occurring via metabolic selection. Infusion of a *Fah* expressing retroviral vector into the portal vein of mutant mice followed by 2 months of *in vivo* selection resulted in Fah expression in >90% of parenchymal hepatocytes and functional correction of the diseased liver.

Liver repopulation by transplanted hepatocytes

To test the hypothesis that Fah expressing hepatocytes have a selective advantage in Fah deficient livers, hepatoctyes from 10-week old congenic wild-type males were isolated and transplanted into recipient female $Fah^{\Delta exon5}$ mutant mice. For the initial experiments 1×10^6 hepatocytes were injected into the spleens of four 8-week old recipients. After transplantation these animals were separated into two groups: 2 recipients were kept on NTBC (group T1), while NTBC was discontinued after transplant in the others (group T2). Two groups of non-transplanted litter mates served as controls: these were 1) wild-type sib-

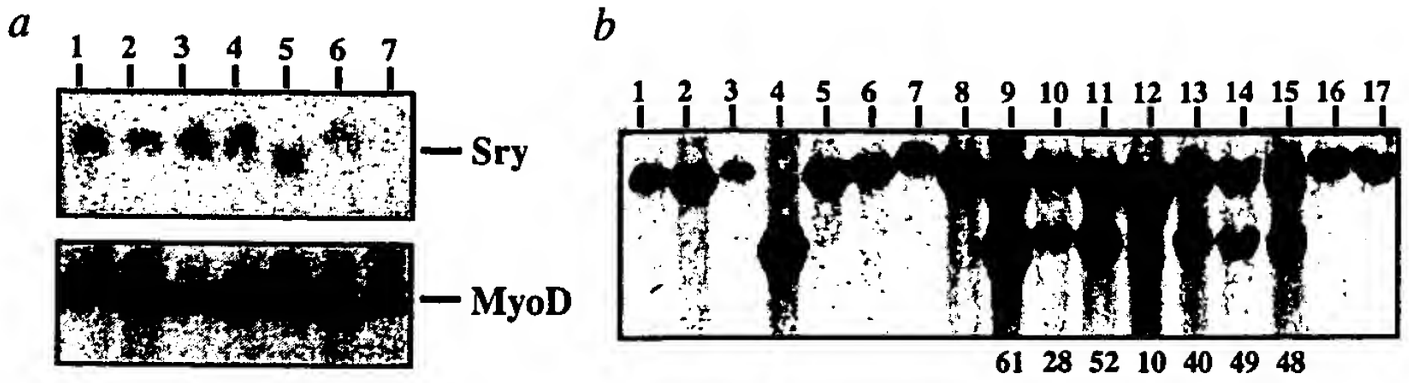

Fig. 2 Southern blot analysis of livers repopulated by transplanted cells. *a*, Genomic DNA isolated from liver probed with murine *sry* (top). The same blot was stripped and rehybridized with *MyoD*. Lanes 1–5 were from female mutant animals transplanted with male wild-type hepatocytes. Samples were obtained 8 weeks after transplant and discontinuation of NTBC. The animals in lanes 1–4 received 10^6 cells, the mouse in lane 5, 10^3 cells. Lane 6 was a male control and lane 7 was from a non-transplanted female control. All repopulated livers contained abundant Y specific signal. *b*, Total liver DNA digested with *Hin*dIII and hybridized with a *Fah* genomic probe which distinguishes the targeted (top band) and wild-type (lower band) alleles. Lane 4, homozygous wild-type control; lane 5, untreated mutant control. Lanes 1–3 and 6–8 were from liver samples obtained 1–3 weeks after stopping NTBC and transplantation, before selection was complete. No wild-type DNA was detectable by Southern blot (detection limit ~10% repopulation) until 4 weeks after transplantation (lane 15). Lanes 9–17 were from mutant animals transplanted with wild-type cells and then taken off NTBC (9–15) or kept on NTBC (16, 17) for 8–10 weeks. The number of transplanted cells were 10^4 (lane 9), 10^3 (lanes 10, 11), 10^2 (lane 12), and 10^6 (lanes 13–17). All mice in which NTBC was discontinued had readily detectable wild-type signal (including mutants which received 1,000 or fewer donor cells). No wild-type signal was present in transplanted mutants on NTBC. The number below lanes

lings and 2) mutant sibs, in which NTBC treatment was also stopped. The weights of both transplant groups and controls were followed weekly. All non-transplanted mutant animals first began losing weight 1 week after stopping the drug and then died within 2 months. In contrast, both groups of transplanted animals and the wild-type controls continued to gain weight (data not shown). The transplanted mice off NTBC gained weight as well as wild-type controls (data not shown). Eight weeks after transplantation the animals were killed and analysed. The macroscopic appearance and weight of transplanted mutants treated with NTBC were indistinguishable from the wild-type controls, demonstrating the protective effect of NTBC on liver function in Fah deficiency[15]. However, the transplanted mice in which NTBC had been stopped also had completely normal macroscopic liver morphology, indicating the possibility of liver repopulation. Next, we determined the levels of Fah enzyme activity in the harvested livers. Transplanted animals off NTBC had Fah levels in the range of wild-type controls (70–110%), but those in which NTBC had been continued had very low activity (<5%) (Table 1). NTBC itself is not an inhibitor of Fah[15]. Therefore, this result suggests that the liver in group T2 was repopulated by transplanted cells with normal Fah activity and that the selection could be blocked by NTBC. To determine the degree of repopulation, we performed immune histology with an Fah antibody (Fig. 1 *a–c*). In all transplanted animals, which were subjected to full selection (no NTBC for 8–10 weeks), >80% of parenchymal hepatocytes stained positive for Fah. The remaining Fah negative hepatocytes tended to cluster around the terminal hepatic veins in zone 3 of liver lobules.

The transplantation experiment with 10^6 donor hepatocytes was repeated multiple times and similar results were obtained each time. In total 14 animals were taken off NTBC after transplantation and 5 were kept on the drug.

The kinetics of liver repopulation was studied by harvesting liver samples from transplanted mutant mice 1, 2, 3, 4, 6 and 8 weeks after transplantation. Fah immunohistology showed the clonal growth of transplanted donor cells (Fig. 1*d*). Wild-type DNA was first detectable by Southern blot 4 weeks after transplantation (Fig. 2*b*); after 8 weeks no further increase in the number of wild-type cells was observed.

Liver repopulation is not due to somatic reversion

In the transgenic urokinase model of liver repopulation, restoration of a wild-type genotype by somatic reversion was frequently observed[20,21]. Hepatocytes which had lost the urokinase transgene were positively selected and formed a repopulation nodule. A spontaneous reversion of the $Fah^{\Delta exon5}$ mutation is unlikely, because this requires an in-frame deletion of the neomycin resistance cassette used in the targeted gene disruption[9]. We have never observed spontaneous survival and restoration of enzyme activity in a Fah mutant animal after discontinuation of NTBC. However, to confirm that the observed repopulation of Fah mutant liver was not due to somatic reversion, we extracted DNA from repopulated livers and performed hybridization of Southern blots with a Y-chromosome specific probe. This analysis was possible because all recipient mice were female and all donor hepatocytes were male. Y specific bands were readily detected in all repopulated animals but not in female controls (Fig. 2*a*). Thus, the repopulation was due to donor hepatocytes and not loss of the targeted Fah mutation.

To quantitate the contribution of the donor to the total liver DNA of transplanted animals, we performed a Southern blot hybridization with an Fah genomic probe, which detects different size bands in mutant and wild-type mice[9]. Probe A hybridizes to a 5.5-kb band in *Hin*dIII digests of wild-type DNA while the targeted FAH allele produces a 6.9-kb band (Fig. 2*b*). In repopulated livers harvested after 8 weeks of selection 40–60% (average 55%) of total liver DNA was donor derived. In mouse, approximately 60% of total liver DNA originates from parenchymal hepatocytes[22] — therefore the figure obtained by Southern blot is consistent with the >80% repopulation observed by immune histology.

Liver repopulation by 1,000 donor cells

We sought to determine the minimum number of transplanted donor cells required to rescue Fah mutant mice and repopulate the recipient liver. To estimate this number, we transplanted increasingly smaller numbers of donor hepatocytes, beginning at 100,000 then descending to 10,000, 1,000, 800, 600, 400 and 100 cells. Transplantations with 10,000 or more cells were done by intrasplenic injection, while the lower numbers of cells were injected directly into

the portal vein. This was done in order to accurately control the number of donor cells which reached the recipient liver. All animals given 10,000 (n = 9) or more donor hepatocytes survived (Table 2). Five of six mutants, which received 1,000 cells survived to 10 weeks post transplantation and had evidence of extensive repopulation by Southern blot analysis, FAH enzyme assay and immunohistology (Figs 1, 2). The survival rate dropped when lower numbers were used, and just 1/6 mice survived when only 100 donor cells were transplanted. Therefore approximately 1,000 donor cells is the minimal number of adult hepatocytes required to repopulate an Fah mutant liver to > 50%. A normal sized mouse liver contains approximately 2–3 × 10^7 cells hepatocytes. If all 1,000 transplanted cells participated equally in the repopulation this expansion would require about 15 cell divisions ($\log_2$ of 3 × 10^7/1,000 = 14.87).

The livers from animals which had received less than 1,000 hepatocytes were only partially repopulated and mosaicism of healthy (red) and diseased (yellow) tissue could be observed in survivors (Fig. 3). In these mice distinct repopulation nodules of 0.5–1.5 mm diameter were visible (Figs 1*b*, 3*a*). Hepatocytes in Fah-expressing areas had normal morphology, whereas directly adjacent mutant tissue showed dysplasia, necrosis and inflammation, demonstrating that the defect in Fah deficiency is cell autonomous (Fig. 1*e*,*f*).

Retroviral gene transfer into Fah-deficient liver

The results obtained with transplantation of wild-type hepatocytes demonstrated that Fah expressing cells had a strong selective growth advantage in the livers of mutant mice, and that as few as 1,000 Fah-expressing cells could achieve a high percentage repopulation. We therefore reasoned that mutant hepatocytes corrected by gene transfer should also have a growth advantage and that transduction of only a fraction of mutant liver cells should result in the eventual repopulation of the diseased liver by healthy cells. Because of the stable integration of retroviral vectors and the resulting permanence of gene expression, we utilized a retrovirus (G1FSvNa) expressing human *FAH* (Fig. 4) from the viral LTR promoter. This promoter is known to remain active long-term *in vivo* in liver[23]. In the first set of experiments, mutant animals received a single injection of virus directly into the portal vein 2 days after a partial hepatectomy or 7 days after discontinuing NTBC. Control animals were infused with a ßgal expressing control virus. None of the control animals (0/14) survived the selection period of 8–12 weeks without NTBC, whereas 52% (29/56) of the animals injected with G1FSvNa survived. No significant difference in survival was detected between mice in which liver regeneration was induced by partial hepatectomy versus by NTBC withdrawal. In both groups *FAH* retrovirus-treated mutant mice lost weight for approximately 4 weeks after injection and then recovered. Mutant mice which had received a single G1FSvNa injection were harvested for analysis 3–5 months after treatment. Mosaic livers were observed in 27/29 surviving animals, consisting of corrected (red) and uncorrected (yellow, nodular) tissue upon macroscopic inspection (Fig. 3). Normal liver appearance was observed in the remaining animals (2/29), indicating that a majority of hepatic tissue had been corrected. Healthy and diseased areas of the retrovirus-treated livers were dissected, DNA and RNA were isolated and samples were processed for immune histology and

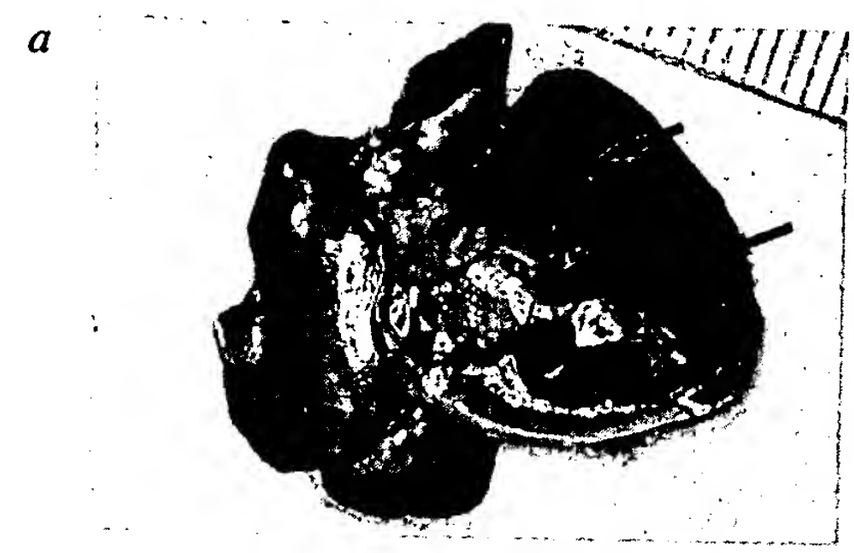

Fig. 3 Whole livers from transplanted and retrovirus treated Fah mutants. *a*, Liver from a mutant animal transplanted with 600 wild-type cells. Red (dark) tissue consists of Fah positive cells, whereas the yellow (lighter colored) tissue is Fah negative. Individual small nodules of healthy tissue (arrows) are visible. Individual nodules measure 0.5–1.5 mm in diameter. An area of coalescent nodules (open arrow) is present. *b*, A single injection of G1FSvNa virus results in a liver mosaic for healthy (dark) FAH-expressing and diseased (light) tissue. *c*, Mutant liver injected multiple times with retrovirus. Macroscopically this specimen is indistinguishable from wild-type controls and has healthy appearing red tissue in all lobes. The ruler in the photographs has 1 mm divisions.

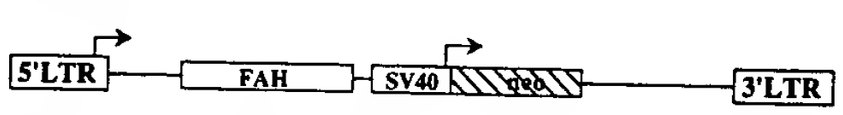

Fig. 4 Structure of the G1FSvNa retrovirus. Arrows indicate initiation sites and the direction of transcription.

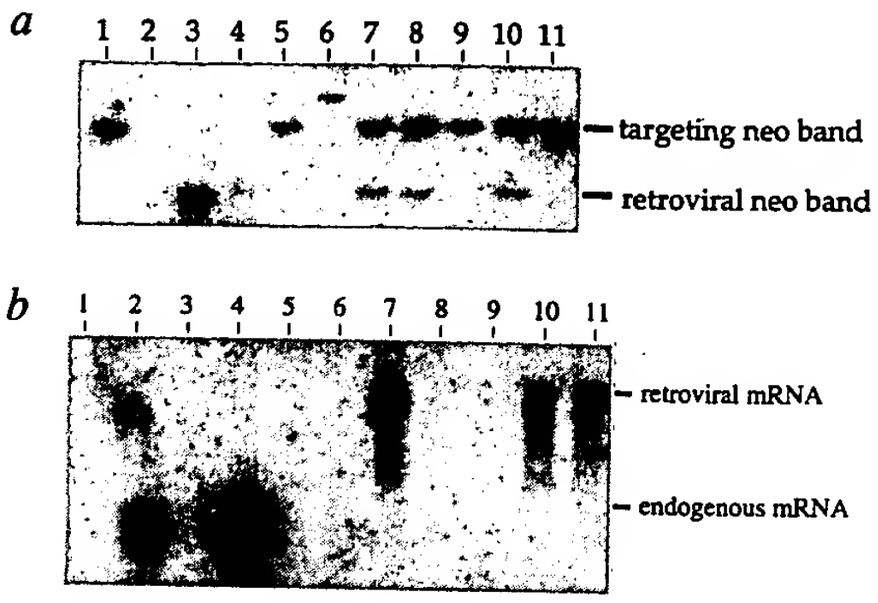

Fig. 5 Southern and northern blot analysis of mutant animals treated by *in vivo* retroviral transduction. *a*, Southern blot of liver genomic DNA digested with *Hin*dIII and *Eco*RI and probed with the neomycin phosphotransferase gene. The top band is derived from the neomycin resistance expression cassette used for gene targeting; the lower band is from the G1FSvNa provirus. Lanes 1 and 11, homozygous mutant control; lane 2, wild-type control; lane 3, packaging cell line DNA; lane 4, packaging cell line diluted 1:10; lane 6, size marker lane; lanes 5, 7, 8 and 9 were from a mutant that received a single dose of the *FAH* retrovirus. Lanes 5 and 9 were from diseased, yellow tissue; lanes 7 and 8 were from adjacent healthy, red liver. A proviral signal was present only in the corrected tissue. Lane 10, total liver DNA from a mutant animal which received 5 G1FSvNa injections. A single copy proviral signal was present throughout all regions of the liver. *b*, Northern blot of 10 µg total liver RNA probed with a murine *Fah* cDNA probe. The upper band is derived from the G1FSvNa retrovirus and the lower band is the endogenous *Fah* band. Lanes 1 and 3, mutant controls; lane 2, heterozygous control; lane 4, wild-type control; lane 5, ß-gal virus treated control; lanes 6, 8 and 9, samples from diseased (yellow) areas in *FAH* retrovirus injected animals; lanes 10 and 11, samples from healthy, FAH-expressing areas of retrovirally treated animals. The FAH expression in these livers is retrovirally derived. No endogenous *Fah* signal is visible.

FAH enzyme assay. In some livers only small nodules of healthy tissue were present, whereas in others entire lobes looked normal. FAH enzyme activities in healthy areas ranged from 2–37% of wild-type levels with a mean of 13% (Table 1). To determine whether the retrovirus was responsible for this FAH expression, DNA was digested with *Eco*RI *and Hin*dIII and probed with the neomycin phosphotransferase gene (Fig. 5*a*). This assay detects a constant 3-kb fragment within the G1FSvNa provirus and a 4.2-kb fragment from the neomycin resistance expression cassette used during gene targeting. Quantitation of the band intensities showed that the average ratio of the provirus band to the knock-out band was about 0.35. A single copy of provirus per hepatocyte would give rise to a ratio of 0.3 because only 60% of total liver DNA is derived from parenchymal hepatocytes and because there are 2 copies of the neo expression cassette in each cell of mutant mice. Thus, FAH expressing nodules on average contained at least one copy of provirus per parenchymal cell. The high level of viral transduction was further confirmed by northern blot analysis (Fig. 5*b*). Virally derived *FAH* mRNA was readily detectable in FAH-expressing areas in quantities approaching endogenous *Fah* expression in wild-type mice. No mRNA of the smaller wild-type size was seen, proving that FAH enzyme activity in these retrovirally treated animals was due to the G1FSvNa virus and not somatic reversion. Immune histology also showed FAH expression in healthy appearing tissue (Fig. 1*h,i*). The areas of expression appeared nodular and clonal and corresponded to normal hepatocellular morphology. In contrast, adjacent non FAH expressing areas showed massive hepatocellular dysplasia and necroinflammation (Fig. 1*i*)

Only partial correction was achieved in most animals with a single retrovirus injection. Therefore, to further optimize gene transfer, a sialastic catheter was implanted in the portal vein of recipient mutant animals. This catheter could be accessed subcutaneously for multiple sequential virus infusions. To induce liver regeneration, recipient mice were either partially hepatectomized ($n = 4$) or NTBC was discontinued ($n = 3$) on day 0. G1FSvNa was infused via the catheter on days 2, 3, 4 and again on days 10 and 11. Animals treated in this fashion gained weight normally despite discontinuation of NTBC. All livers had normal macroscopic appearance at harvest 8–12 weeks after the retrovirus injection and no diseased tissue was visible. FAH enzyme activities in such livers ranged from 20–80% of wild-type with a mean of 59% (Table 1). Southern blot analysis demonstrated the presence of at least 1 provirus/cell throughout the entire liver (Fig. 5*a*). The macroscopic inspection was confirmed by histology and immune histology. The majority of hepatocytes (>80%) in mutant livers, which had received multiple doses of the retrovirus were now FAH positive (Fig. 1*h*). The lobular architecture and hepatocellular morphology of corrected areas was normal. Within FAH positive tissue, nodular areas of high, medium and low FAH expression could be distinguished (Fig. 1*h,i*). These probably represent clones derived from a single FAH transduced precursor, in which the level of FAH expression depended on the proviral integration site and/or proviral copy number.

FAH immunohistology in two multiply injected animals harvested 48 hours after the last retroviral injection showed fewer than 1% of hepatocytes staining positive at that time (data not shown). Therefore, the high level of tissue correction obtained was due primarily to selection of FAH transduced cells rather than high efficiency of the initial gene transfer.

Liver function in retrovirus-treated mice

The gene transduction of a majority of hepatic parenchyma and the high levels of FAH expression achieved suggested that this might be sufficient to obtain significant improvement or possibly total correction of overall liver function. Several parameters of liver function were analysed and plasma levels of one of the toxic metabolites, succinylacetone (SA), were measured at the time of sacrifice. The plasma levels of aspartate aminotransferase (AST), a parameter of hepatocyte integrity, and conjugated bilirubin, a measure of liver synthetic and secretory function, were restored to normal levels in mutant mice transplanted with large numbers of hepatocytes and in those which had received multiple retrovirus infusions (Table 1). In contrast, both these parameters were abnormally elevated in untreated controls and in mice, which had received only one G1FSvNa injection. SA is derived from the toxic metabolites maleylacetoacetate and fumarylacetoacetate, which accumulate in HT1. Multiple virus injections or hepatocyte transplantation

also corrected plasma SA levels. The improved liver function tests and the good weight gain of treated animals indicated that a substantial therapeutic benefit was derived from the gene transfer procedure.

Discussion

***In vivo* selection as a principle in liver gene therapy.** Our results have implications for hepatic gene therapy, liver stem cell biology and the treatment of human HT1. Previous liver repopulation experiments performed in transgenic urokinase mice have documented the high regenerative potential of adult mouse hepatoctyes[21]. In those studies ~1×10^5 donor cells were injected intrasplenically into the diseased livers of recipient animals and gave rise to 30–80% repopulation, due to the selective growth advantage of wild-type cells. Calculations based on the average size of regenerative nodules indicated that the repopulating cells had divided 12–16 times. Here we have shown that this growth potential can be used to replace diseased liver tissue in a model of genetic human liver disease. As few as 1,000 donor cells (0.003% of the total number of hepatocytes in an adult mouse liver) injected directly into the portal circulation were sufficient to replace the majority of cells in an Fah-deficient liver by multi-nodular repopulation. For this expansion 14–15 mitotic cell divisions would be required if all cells participated equally in the repopulation. Because of the average size of nodules (about 1–1.5 mm) and the degree of repopulation, we conclude that a substantial portion of the 1,000 transplanted cells (at least 10%) participated in the regeneration and had high regenerative capacity. The experience with somatic reversion in human HT1 and expansion of FAH positive nodules[18,19] suggests that human hepatocytes have a similar potential for multiple cell divisions. In our model we showed that this phenomenon can be exploited therapeutically and that replacing only a small fraction of diseased hepatocytes may result in significant organ repopulation.

Many different strategies have been used in attempts to achieve high levels of permanent gene expression in liver gene therapy. Our results show that standard retroviral vectors can achieve this goal, when the high regenerative potential of transduced hepatocytes and *in vivo* selection are utilized. Current human liver gene therapy trials utilize *ex vivo* retroviral gene transfer, followed by autologous transplantation of the transduced cells[24]. In humans and large animals these protocols are able to achieve gene transfer into 0.1–1% of hepatocytes[24–26] and thus only 6–10 cell divisions with positive selection could reconstitute the majority of the diseased organ. This raises the hope that human HT1 may be curable by already established *ex vivo* retroviral gene therapy procedures.

There are three requirements for *in vivo* selection in liver: i) wild-type cells must have a growth advantage over mutant cells, ii) liver regeneration must be occurring and iii) the defect has to be cell autonomous injuring mutant hepatocytes, but not neighboring wild-type cells. These principles clearly apply in HT1, but there are a number of other hereditary liver disorders, in which toxic metabolites accumulate in the hepatocyte and are unlikely to poison neighboring cells. A partial list of these includes galactosaemia, hereditary fructose intolerance, the glycogen storage diseases, Wilson's disease, bile acid synthesis defects and z-allele α1-antitrypsin deficiency[2]. Active liver regeneration is not present in all of these, but could easily be induced artificially by exogenous liver injury in order to allow expansion of transplanted wild-type cells or hepatocytes corrected by gene therapy. Some acquired chronic liver disorders such as hepatitis B and C infection may also be amenable to this approach. Gene transfer would be used to generate a population of hepatocytes resistant to infection by the hepatitis virus allowing them to compete for repopulation of the diseased liver.

The tyrosine catabolic pathway may possibly have general use for *in vivo* metabolic selection of genetically transduced hepatocytes in non Fah-deficient animals. Gene therapy vectors which co-express a gene of interest and *Fah* as a selectable marker could potentially be used to enrich for transduced cells, if the tyrosine catabolic pathway was stressed by substrate overload (homogentisic acid) or by pretreatment of pharmacological inhibitors of Fah. Only parenchymal hepatocytes express all the steps of tyrosine degradation and thus toxicity from such a strategy would likely be limited only to this cell type and have few systemic side effects.

In vivo selection for genetically transduced cells may also be useful in organs other than liver. Recent evidence suggests that gene corrected T-cells have a selective growth advantage in adenosine deaminase deficiency[27].

The effects of liver repopulation on cancer risk. In our model *Fah* gene therapy was effective in restoring normal liver function, but in all livers analysed a sizable proportion of Fah-deficient hepatocytes (5–10%) remained after 2–3 months. Malignant transformation is common in human HT1[28] and also in murine Fah deficiency[15]. Thus, it is too early to judge whether *in vivo* retroviral gene transfer truly represents a 'cure' for the disorder and is therapeutically equivalent to liver transplantation[17]. Retrovirally treated and transplanted animals will have to be followed long-term to determine the cancer incidence and also to assess whether renal function is stabilized by hepatic expression of Fah. Tumour progression in general probably requires both genetic instability (to allow for the development of additional growth promoting mutations) and cell division (to allow for selection of these mutations). After repopulation with Fah-expressing cells the livers of treated animals are no longer regenerative and the rate of mitosis of all cells (mutant and wild-type) is greatly reduced. This reduction in cell turnover may prevent or delay the development of hepatocellular carcinoma, even in the presence of residual Fah-deficient cells.

A model system for liver stem cell biology. Many basic questions about liver regeneration in various disease states remain unanswered at the current time. While it is clear that a hepatic stem cell capable of multilineage differentiation exists during embryonic development[29], the existence of liver stem cells in adult life is disputed[30]. The liver nodule forming assay in $Fah^{\Delta exon5}$ mice represents a novel system for the study of liver stem cell biology. So far, the urokinase transgenic mouse is the only other similar system described[21,22], but the

Fah-deficient mouse reported here has several significant advantages for such experiments. First, spontaneous somatic reversions have never been observed. Second, the use of NTBC provides and 'on and off' switch for selection. Because of this mutant animals can be kept healthy until adulthood, which facilitates surgical manipulations. Selection can also be accelerated by administration of homogentisic acid, a precursor of fumarylacetoacetate. Third, and most significantly, the selection in Fah deficiency is positive — gain of a function — whereas the selection in urokinase transgenics is negative — for transgene loss. Because of this, Fah-deficient hepatocytes can be marked by a retrovirus and then positively selected. This feature will permit the tracking of individual cells over time and will allow us to ask the question whether marked precursor cells can give rise to more than one hepatic cell type.

Many properties of the haematopoietic stem cell system were delineated in repopulation experiments which utilized the isolation and transplantation of distinct cell populations from bone marrow[31]. Retrovirus marking has become a standard tool in studies of cell fate and developmental biology[32,33]. Both of these approaches can now be applied to liver regeneration. Hopefully this new experimental tool will aid in the resolution of the some of the unanswered questions in liver biology.

Methods

Strains of mice and animal husbandry. We utilized the *Fah*$^{\Delta exon5}$ strain mice as described[9]. All transplantation experiments were performed with congenic mice of the 129Sv background. Retrovirus experiments were performed with 129Sv/C57BL hybrid mice. All breeders and all mutant animals were treated with NTBC containing drinking water at a concentration of 7.5 mg/l (provided by S. Lindstedt, Gøtheborg, Sweden). This provides an approximate dose of 1 mg/kg body weight per day. For genotyping, PCR was carried out with a 3 primer PCR on 200 ng tail-cut DNA as described[9]. Animal care and experiments were all in accordance with the Guidelines of the Department of Animal Care at Oregon Health Sciences University.

Hepatocyte transplantation and selection. Parenchymal hepatocytes were isolated from congenic male wild-type animals by a two step collagenase perfusion[34]. Cell number and viability were determined by trypan blue exclusion in a haemocytometer. The appropriate number of donor cells were resuspended in 100 µl of Dulbecco's minimal essential media (DMEM) (Gibco) with 15% fetal calf serum (FCS) and injected intrasplenically[35] or directly into the portal vein of mutant female recipient animals. All mutant mice were kept on NTBC until the time of transplantation or retrovirus infusion. NTEC was discontinued 2 d after the last therapeutic intervention to permit positive selection. The weight of experimental animals was measured weekly.

Retrovirus treatments. We utilized the retroviral vector G1FSvNa generated by cloning the human *FAH* cDNA[36] into the *Sal*I restriction site of the pG1XSVNa construct[37]. This virus contained the human *FAH* gene driven by the retroviral LTR promoter and the selectable marker neomycin phosphotransferase driven by the SV40 promoter. A high titer producer cell line was generated by electroporation of the viral plasmid into the ecotropic packaging cell line gp+E 87 (ref. 38). After G418 selection supernatants from the ecotropic producer were used to multiply infect the amphotropic packaging line gp+E Am12 (ref. 39). A clone (A18) producing 1×10^6 viral particles/ml of supernatant was identified and used for all subsequent infections. A18 cells were grown in high glucose DMEM and 10% bovine calf serum and 12 ml of viral supernatant were harvested from confluent 150 mm dishes. Supernatant was harvested every 24 h, filtered through 0.2 µm filters and frozen at –80 °C. For *in vivo* infection the virus was thawed and then concentrated 10 fold by centrifugation in a Centriplus 100 (Amicon) molecular weight cut-off column. After concentration, viral titers were ~1×10^7/ml when measured on 3T3 cells.

Several different protocols were used for *in vivo* retroviral gene transfer. Liver cell regeneration was induced by either 2/3 partial hepatectomy or by withdrawing NTBC treatment on d 1. All protocols involved the direct injection of viral supernatant into the liver via the portal vein. The vein was cannulated either with a 30 gauge needle for a one time injection or a permanent catheter was inserted for multiple administrations[40]. The catheter could be accessed subcutaneously. Concentrated viral supernatant (0.6 ml) with 8 µg/ml polybrene was injected over 30 min. Single virus injections were done 48 h and multiple injections were performed on d 2, 3, 4, 10 and 11 after partial hepatectomy or NTBC withdrawal.

Southern and northern blots. Liver DNA[41] and RNA[42] were isolated from fresh or liver frozen at –80 °C and Southern and northern blots were performed according to standard protocols[43]. For the detection of male specific DNA, a 380-bp fragment of the murine *Sry* gene[44] was used on *Hin*dIII digested DNA. A *MyoD* cDNA[45] was used as an autosomal control probe. Ratios of wild-type to *Fah*$^{\Delta exon5}$ DNA were determined by hybridizing with probe A, a 900-bp genomic fragment from the mouse *Fah* gene[9]. A Beckman SI Phosphoimager was used to quantitate relative band intensities. Northern blots were probed with a fragment of mouse *Fah* cDNA (nt 791–1103). This fragment is 97% conserved between mouse and human[46] and detects *FAH* mRNA from both species equally well.

Biochemical analysis. Samples from animals were obtained as follows: Animals were killed by decapitation and blood collected by dabbing the wound onto Parafilm. For anticoagulation, the blood was immediately mixed with 10 µl of Na-heparin using a Pipetman. The red blood cells were removed by a brief centrifugation and the plasma was frozen at –80 °C. 20 µl of plasma were mixed with 80 µl of a solution of 7% bovine serum albumin and assayed for AST, bilirubin and creatinine levels with a Kodak Ektachem 700 chemistry analyser. Plasma succinylacetone levels in plasma were measured by a δ-aminolevulinate dehydratase inhibition assay[47]. Fah enzyme assays were carried out at 30 °C on a cytosolic fraction of homogenized liver as described[48]. FAA, the substrate for the assay, is not commercially available and was prepared enzymatically from homogentisic acid as described[48]. Protein concentrations were measured with a Bio-RAD kit[49].

Histology and immune histology. Liver tissues fixed in 10% phosphate-buffered formalin, pH 7.4, were dehydrated in 100% ethanol and embedded in paraffin wax at 58 °C. Four micron sections were re-hydrated and stained with Hematoxylin-Eosin and with a polyclonal rabbit antibody to rat Fah[50,51]. The antibody was diluted in phosphate buffered saline pH 7.4 and applied at concentrations of 1:300,000 at 37 °C for 30 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 and methanol. Avidin and biotin pretreatment was used to prevent endogenous staining. The secondary antibody was biotinylated goat anti-rabbit IgG used at 1:250 dilution (VectorLabs). Color development was performed with the AEC detection kit from Ventana Medical Systems.

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